MBMG
Metabarcoding & Metagenomics

Metabarcoding and Metagenomics 7: 247–262 (2023)
DOI: 10.3897/mbmg.7.96780

Applied Study

Fish diversity assessed by eDNA detection methods in the Rioni River

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Abstract

Due to anthropogenic influences, habitat degradation and a continuous loss of biodiversity in freshwater ecosystems are occurring on a large scale, while these ecosystems constitute invaluable natural resources. Therefore, it is essential to study and monitor freshwater ecosystems to guide conservation efforts. Freshwater ecosystems are one of the less-studied fields in Georgia. Studies about the species distribution of many taxa and/or regions carried out during the last century have not been updated for decades. Here, we report the results of an environmental DNA (eDNA) metabarcoding exercise, based on samples collected from the Rioni River, a tributary to the Black Sea and a crucial aquatic ecosystem regionally and globally. The only comprehensive review of the fish of the Rioni River dates back to 1956. We compared the eDNA-based taxonomic composition to the known faunal composition within the Rioni River and found that the eDNA-based taxonomic coverage approached 75% of the expected total fish fauna. A number of new species occurrences were also found, including the first detection of three invasive alien species (*Carassius gibelio*, *Pseudorasbora parva*, *Rhinogobius lindbergi*) in the Rionis River Basin and a new country record of the ninespine stickleback (genus *Pungitius*) for Georgia. In spite of the usefulness of the eDNA metabarcoding approach, the sparsity of the fish DNA barcode reference library for the region emerged as a limitation to this study. However, our findings still represent a great leap forward in updating fish status on the Rioni River and testing the effectiveness of the eDNA sampling for aquatic species.

Key words: Caucasus, eDNA, fish diversity, Rioni River

OPEN ACCESS

Academic editor: Katy Klymus
Received: 30 January 2023
Accepted: 5 September 2023
Published: 18 October 2023

Citation: Beridze T, Japoshvili B, Edisherashvili T, Anderson C, Mumladze L (2023) Fish diversity assessed by eDNA detection methods in the Rioni River. Metabarcoding and Metagenomics 7: e96780. https://doi.org/10.3897/mbmg.7.96780

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Introduction

Even though the Republic of Georgia is a part of the internationally-recognised Caucasus Biodiversity Hotspot, harbouring tertiary relic flora and fauna (Milne and Abbott 2002; Mittermeier et al. 2004; Habel et al. 2019), its biodiversity is still poorly characterised and conservation measures are needed to protect this diversity (Mumladze et al. 2020). Within Georgia, the Rioni River is one of the largest rivers in the Black Sea Basin, housing the last remaining eastern Black Sea breeding populations of at least three sturgeon species (Beridze et al. 2022a, b) and, thus, is a critical habitat for the conservation of this most

endangered vertebrate group globally (IUCN). This alone makes the Rioni River a focus of global attention. In addition to threatened sturgeon taxa, the Rioni and its tributaries are home to a number of endemic fish species from the Colchic refugium (*Rhodeus colchicus*, *Barbus rionicus*, *Oxynoemacheilus phasicus* etc), for which the Rioni River and its tributaries encompass a major part or entirety of a species' distribution (Bogutskaya and Komlev 2001; Baycelebi et al. 2015; Freyhof et al. 2021). As such, the Rioni is of enormous importance for aquatic biodiversity in the Caucasus. At the same time, the river is subject to ongoing heavy anthropogenic pressure, such as hydro-power development, pollution, mining and poaching (Caruso et al. 2012; Japoshvili et al. 2021; Suciu et al. 2021a). For the conservation of aquatic biodiversity in the face of these challenges, ongoing species monitoring in the Rioni watershed is essential. However, the last comprehensive assessment of the Rioni River fish community is more than half a century old (Elanidze 1956) and, since then, only occasional sampling directed mainly at the biology of an individual fish species has taken place (Levin et al. 2018; Epitashvili et al. 2020; Freyhof et al. 2021).

In the past decade, metabarcoding of environmental DNA (eDNA) has become a promising technique for effective biodiversity monitoring in fresh and marine waters (Bohmann et al. 2014; Pfleger et al. 2016; Cristescu and Hebert 2018). The applications of eDNA techniques include the evaluation of species richness and the monitoring of rare and threatened species (Thomsen et al. 2012; Shaw et al. 2016; Evans and Lamberti 2018). Moreover, eDNA methods are able to detect species that are otherwise difficult to find with traditional sampling (Thomsen et al. 2016; Suarez-Menendez et al. 2020) or can be used for early detection of invasive species (Ficetola et al. 2008; Goldberg et al. 2011). It is already clear that eDNA methods are amongst the most cost-effective for biodiversity monitoring (Rees et al. 2014; Thomsen and Willerslev 2015). Publicly-available and ever-increasing DNA reference libraries, such as GenBank (Benson et al. 2012) or BOLD systems (Ratnasingham and Hebert 2007) are crucial to the usefulness of eDNA technology. However, the lack of reference sequence barcode data in many parts of the world still impedes the effective use of the eDNA metabarcoding approach in those areas. Thus, a concerted effort to improve the eDNA technology and availability of relevant infrastructure and also develop regional DNA barcode inventories is necessary to advance DNA-based biodiversity studies, which will in turn allow for more cost effective, accurate and wider-ranging biodiversity assessments and monitoring (Pawlowski et al. 2018; Weigand et al. 2019).

Despite being a biodiversity hotspot, Georgia and the Caucasus ecoregion as a whole still lack effective biodiversity inventory and monitoring programmes, based on both traditional collection methodologies and new technologies. Thus, our goal was to set a precedent in the Caucasus biodiversity hotspot by using modern techniques in biodiversity inventorying, while also evaluating the effectiveness of the eDNA sampling in assessing the diversity of fish species in the Rioni River.

In the present study, we provide the first eDNA analysis results, based on water samples collected in the Rioni River and compare the obtained data on fish species diversity to those known from literature based on 20th century collections (Elanidze 1956) and to the updated species list of fishes of Georgia (Kuljanishvili et al. 2020, 2021). We demonstrate the utility of eDNA technology to deliver fish biodiversity

information, from a region that lacks records of DNA barcodes for native species with the exception of recent work by (Epitashvili et al. 2020). Due to the success of our pioneering eDNA work in the Rioni River, we encourage further eDNA-based research on aquatic ecosystems in the Caucasus biodiversity hotspot.

Materials and methods

Study area and sampling

The Rioni River is the longest river in Georgia (length – 327 km, annual discharge – 13.37 km^3) and its diverse freshwater community includes a number of endemic fish taxa unique to the region. Along the Rioni River, there are a number of artificial constructions, some of which are insurmountable barriers for freshwater animals. Industrial development of the Rioni River has led to habitat degradation, fragmentation and loss, with artificial barriers formed by dams and weirs posing a particular threat to migratory and diadromous species. One such barrier is the Vartsikhe Hydropower Plant. Along with other anthropomorphic pressures (e.g. poaching, gravel mining, pollution), this dam has significantly reduced the historical spawning area of Black Sea sturgeons from ca. 90 km to 9 km downstream of the River (Suciu et al. 2021b). As a result, sturgeon populations are now on the verge of extinction.

To mitigate the risk of sturgeon extinction in the Rioni and improve their habitat quality, a number of projects have been initiated. One of those projects, led by Fauna & Flora International (FFI), investigated different aspects of surviving sturgeon populations and habitats (Suciu et al. 2021b; Beridze et al. 2022b). As part of this initiative, FFI piloted eDNA sampling between 2018 and 2019 to detect sturgeon species in the river. Water samples were collected during September 2018 and March 2019 from the river mouth to approximately 90 km upstream (Fig. 1). As part of the effort to detect sturgeon, these samples simultaneously generated a catalogue of non-targeted species whose DNA was present in the samples, providing a snapshot of species assemblages at the various sampling locations.

In total, 12 water samples each up to 0.8-litre volume were collected using the NatureMetrics eDNA filter kits. Using a polyethersulphone filter with a 0.8 µm pore size, water was filtered and eDNA preserved according to the manufacturer's protocol (NatureMetrics, UK). The specific volume of water used for each sample was dictated by water turbidity (minimum 150 ml, maximum 800 ml). More precisely, high turbidity precluded higher volumes (Table 1). Samples were collected mostly from the water surface and, in two cases, at a depth of 3 m. Field control samples were not collected for the survey. Samples were shipped to and processed by NatureMetrics for eDNA metabarcoding using the "eDNA Survey – Fish" pipeline (NatureMetrics, UK).

DNA processing

Samples were processed by NatureMetrics company, following the eDNA survey – Fish pipeline, including DNA extraction, amplification, sequencing and DNA analysis. DNA was extracted from 12 filters using a DNeasy Blood and Tissue Kit (Qiagen). PCR inhibitors were removed from extracted DNA using DNeasy

Table 1. The volume of water filtered and the resultant concentration of purified DNA and index PCRs.

Sample ID	Sampling data	Coordinates	Sampling depth	Filtered volume	DNA (ng/µl)	Index (ng/µl)	Species
1	15-Jun-2019	42.14962, 41.68107	3 m	150 ml	> 20	11.1	1
2	22-Mar-2019	42.14962, 41.68107	3 m	250 ml	> 20	17.6	15
3	22-Mar-2019	42.21298, 41.79929	Surface	500 ml	5.26	17.6	24
4	31-Oct-2018	42.20775, 41.80520	Surface	450 ml	2.86	10.2	22
5	25-Sep-2018	42.20504, 41.80986	Surface	500 ml	9.6	4.46	24
6	22-Mar-2019	42.15894, 42.16789	Surface	500 ml	6.26	19.8	22
7	31-Oct-2018	42.14581, 42.18570	Surface	550 ml	0.842	11	24
8	24-Sep-2018	42.14491, 42.18603	Surface	500 ml	4.36	3.26	21
9	23-Apr-2019	42.11546, 42.29542	Surface	650 ml	11.2	12.7	23
10	22-Mar-2019	42.14172, 42.28985	Surface	800 ml	3.04	11.4	19
11	22-Mar-2019	42.11837, 42.33069	Surface	750 ml	6.06	18.6	19
12	21-Mar-2019	42.15686, 42.38307	Surface	750 ml	5.84	10.8	24

PowerClean Pro Cleanup Kit (Qiagen). A hypervariable 12S rRNA gene fragment was amplified in twelve PCR replicates using vertebrate primers with expected 140–200 bp amplicon sizes, excluding primers (Miya et al. 2015). Both negative and positive controls were used alongside all PCRs. The mock community with a known African fish species composition was used, as such species were not expected in the region. No contamination between the mock community and analysed samples was detected. Amplification products were checked on gel electrophoresis. All PCR replicates were pooled and purified and adapters were added before sequencing. The success of this step was checked using gel electrophoresis and quantified using a Qubit high-sensitivity assay. The index PCRs were pooled into the final library and sequenced on Illumina MiSeq V.2 kit at 12 pM with a 10% PhiX spike in. Sequence data were processed using a custom bioinformatics pipeline developed at NatureMetrics (NatureMetrics, UK) passing though quality filtering, dereplication, denoising and taxonomic assignment steps. The bcl2fastq software was used for demultiplexing the sequences and USEARCH (Edgar 2010) was used for merging paired-end FASTQ reads for each sample. Primers were removed from the forward and reverse reads using cutadapt (Martin 2011). Sequences between 140–200 bp length were retained in the analysis after removing primers. Sequences with an expected error rate per base of 0.05 or below were quality filtered using USEARCH (Edgar 2010) and were dereplicated. Unique reads were denoised using UNOISE (Edgar 2016). ZOTUs (zero-radius OTUs) were clustered at 99% similarity with USEARCH. All dereplicated reads were mapped for each sample to the ZOTU representative sequences at 97% identity threshold. Taxonomy assignments were made via BLAST (Altschul et al. 1990; Camacho et al. 2009) searches of the representative sequences against the NCBI nucleotide database and with the custom in-house taxonomic database of 12S fish sequences at the NatureMetrics (NatureMetrics, UK). Identifications of the sequences were based on the highest available percentage sequence identity with a minimum e-score of 1e-20 and a hit length of at least 80% of the query sequence. For the species-level

identification, a sequence identity cut-off of 97% was used. Confident species IDs were made at ≥ 98%, sequences between 97 and 98% similarities were retained for species identification and interpreted, based on local knowledge. In case of multiple hits meeting these criteria, more conservative higher taxonomic classification was selected. Low abundance detections (< 0.05% or < 10 reads) per sample were excluded. All samples were pooled together and summarised, based on their taxonomic assignments. The OTUs identified as originating from human, food or livestock were removed from the database.

Results

DNA sequences

The average total DNA yield from samples was 7.94 ng/µl and ranged from 0.842 ng/µl (Tsilori Oct 18, Sample ID #7-Table 1) to > 20 ng/µl (Market Bridge Mar 19, Sample ID #12 and Poti Market Bridge, Sample ID #5-Table 1). Amplification was successful for all 12 samples. A total of 747,622 high-quality sequences were generated and used for taxonomic assignment.

Sample composition

A total of 34 fish taxa were detected across the 12 samples (excluding non-metazoan and contaminant taxa), of which 22 could be confidently identified to species level (Table 3). The remainder were identified at the above/species taxonomic level. The identified fish species belong to two classes, 11 orders, 15 families and 32 genera (Table 2). The average species richness (per sample) was 20 and ranged from 1 (location 1) to 24 (localities 3, 5, 7 and12) and the diversity is summarised in Table 1. A nase species belonging to the genus *Chondrostoma* (possibly *C. colchicum* or *C. cyri*), accounting for 18% of the total sequence reads was the most abundant in terms of DNA sequences.

In the entire dataset, DNA of four alien species was detected: (1) rainbow trout (*Oncorhynchus mykiss*); (2) mosquitofish (*Gambusia holbrooki*); (3) bighead carp/silver carp (*Hypophthalmichthys nobilis*/*H. molitrix*) and; (4) grass carp (*Ctenopharyngodon idella*). In addition, there were a number of other species (eight in total) that we initially identified as non-native. However, these taxa are most probably native species of the Rioni River, closely related to other congenerics represented in the NatureMetrics reference database (Table 2). For example, we detected barbel (*Barbus barbus*) at four locations, but this species is not listed amongst the Georgian fish according to Kottelat and Freyhof (2008) and Kuljanishvili et al. (2021). Only schneider – *Alburnoides* sp. was detected in every sample. Nine other species were detected in 11 samples: Barbel (*Barbus* sp.), Khramulya (*Capoeta capoeta*), Prussian carp (*Carassius gibelio*), Nase (*Chondrostoma* sp.), Goby (*Ponticola* sp.), Topmouth gudgeon (*Pseudorasbora parva*), Bitterling (*Rhodeus* aff. *colchicus*), Roach (*Rutilus rutilus*) and Chub (*Squalius cephalus*). No sturgeon was detected in any sample, while only Schneider (*Alburnoides* sp.) was found in the Poti Market Bridge sample (location 1) with a significant number of palmate newt (*Triturus helveticus*) and edible dormouse (*Glis glis*) sequences. The lower diversity of fish DNA detected here is probably due to the smaller volume of water filtered.

Table 2. Species composition in the Rioni River according to Elanidze (1956) and after eDNA investigation. Species names according to modern taxonomy are given in the first column. Note that some of the species were not recorded neither by Elanidze (1956) nor by eDNA survey, but were known from other sources (indicated by asterisk).

Taxonomy according to Kuljanishvili et. al. (2020, 2021); Epitashvili et al. (2020)	Records by Elanidze (1956)	Detected by eDNA
Anguillidae		
1. *Anguilla anguilla*[1]	–	–
Acheilognathidae		
2. *Rhodeus colchicus*	as *R. sericeus*	as *R. sericeus*
Acipenseridae		
3. *Huso huso*	as *H. huso*	–
4. *Acipenser nudiventris*	as *H. nudiventris*	–
5. *Acipenser gueldenstaedtii*	as *H. gueldenstaedtii*	–
6. *Acipenser sturio*	as *H. sturio*	–
7. *Acipenser stellatus*	as *H. stellatus*	–
Atherinidae		
8. *Atherina caspia*	as *A. mochon*	–
Carangidae		
9. *Trachurus mediterraneus*	–	as *T. mediterraneus*
Clupeidae		
10. *Alosa tanaica*	as *Caspialosa paleostomi*	–
Cobitidae		
11. *Cobitis satunini*	as *C. taenia*	as *Cobitis* sp.
Cyprinidae		
12. *Barbus rionicus*	as *B. tauricus*	as *B. barbus*
13. *Capoeta sieboldii*	as *Varicorhinus sieboldii*	as *C. capoeta*
14. *Cyprinus carpio*	as *C. carpio*	as *C. carpio*
15. *Carassius gibelio*	–	as *Carassius* sp.
Engraulidae		
16. *Engraulis encrasicolus*	–	as *E. encrasicolus*
Esocidae		
17. *Esox lucius*	as *E. lucius*	as *E. lucius*
Gobiidae		
18. *Babka gymnotrachelus*	as *Mesogobius gymnotrachelus*	–
19. *Ponticola constructor*	as *Neogobius* (*C.*) *constructor*	as *Ponticola* sp.
20. *Neogobius melanostomus*	as *N. melanostomus*	–
21. *Neogobius fluviatilis*	as *N. fluviatilis*	as *N. fluviatilis*
Gobionidae		
22. *Gobio artvinicus*	as *G. gobio*	as *G. gobio*
23. *Pseudorasbora parva*	–	as *P. parva*
Leuciscidae		
24. *Petroleuciscus borysthenicus*	as *Leuciscus borysthenicus*	–
25. *Leuciscus aspius*	as *Aspius aspius*	as *Leuciscus* spp.

Taxonomy according to Kuljanishvili et. al. (2020, 2021); Epitashvili et al. (2020)	Records by Elanidze (1956)	Detected by eDNA
26. *Chondrostoma colchicum*	as *C. colchicum*	as *C. nassus*
27. *Alburnus derjugini*	as *Chalcalburnus chalcoides*	as *A. chalcoides*
28. *Alburnus alburnus*	as *A. alburnus*	as *A. alburnus*
29. *Alburnoides fasciatus*	as *A. bipunctatus fassciatus*	as *A. bipunctatus*
30. *Blicca bjoerkna*	as *B. bjoerkna*	–
31. *Abramis brama*	as *A. brama*	as *A. brama*
32. *Rutilus* spp.	as *R. rutilus*	as *R. rutilus*
33. *Squalius orientalis*	as *Leuciscus cephalus*	as *S. cephalus*
34. *Scardinius erythrophthalmus*	as *S. erythrophthalmus*	–
35. *Vimba vimba*	as *V. vimba*	as *V. vimba*
Mugilidae		
36. *Mugil cephalus*	as *M. cephalus*	as *M. cephalus*
37. *Chelon auratus*	as *Mugil auratus*	–
38. *Chelon saliens*	as *Mugil salines*	–
Nemacheilidae		
39. *Oxynoemacheilus phasicus*[2]	as *Nemachilus* sp.	–
Oxudercidae		
40. *Rhinogobius lindbergi*	–	+
Petromyzontidae		
41. *Lampetra ninae*[3]	–	as *Lampetra* sp.
Percidae		
42. *Sander lucioperca*	as *Lucioperca lucioperca*	–
43. *Perca fluviatilis*	as *P. fluviatilis*	as *P. fluviatilis*
44. *Gymnocephalus cernua*	–	–
Poeciliidae		
45. *Gambusia holbrooki*	as *G. affinis*	as *G. holbrooki*
Salmonidae		
46. *Salmo labrax*	as *S. fario* and *S. labrax*	as *S. labrax*
47. *Oncorhynchus mykiss*	–	as *O. mykiss*
Scombridae		
48. *Scomber scombrus*	–	as *S. scombrus*
Siluridae		
49. *Silurus glanis*	as *S. glanis*	as *S. glanis*
Syngnathidae		
50. *Syngnathus abaster*	as *S. nigrolineatus*	–
Pleuronectidae		
51. *Platichthys flesus*	as *P. flesus*	–
Xenocyprididae		
52. *Ctenopharyngodon idella*	–	as *C. idella*
53. *Hypophthalmichthys nobilis/molitrix*	–	as *H. nobilis/molitrix*
Gasterosteidae		
54. -	–	as *Pungitius pungitius*

[1,3]Elanidze (1983); [2]Freyhof et al. (2021); [4]Epitashvili et al. (2020).

Table 3. Species DNA sequence representation in each of the 12 water eDNA samples collected from September 2018 to March 2019. Species names are given after adjusting the NatureMetrics results to the up-to-date fish list of south Caucasus provided by Kuljanishvili et al. (2020) and subsequent literature.

Species\Samples	1	2	3	4	5	6	7	8	9	10	11	12
Engraulis encrasicolus	x	x									x	
Cobitis satunini	x		x			x	x	x	x		x	
Abramis brama		x		x	x			x	x			
Alburnoides fasciatus	x	x	x	x	x	x	x	x	x	x	x	x
Alburnus alburnus		x		x	x			x	x	x	x	
Alburnus derjugini	x		x	x	x	x	x	x	x	x		
Barbus rionicus	x	x	x	x	x	x	x	x	x	x	x	
Capoeta sieboldii	x	x	x	x	x	x	x	x	x	x	x	
Carassius gibelio	x	x	x	x	x	x	x	x	x	x	x	
Chondrostoma colchicum	x	x	x	x	x	x	x	x	x	x	x	
Ctenopharyngodon idella	x		x			x	x	x			x	
Cyprinus carpio	x		x	x		x	x	x	x		x	
Gobio artvinicus				x	x			x	x		x	
Hipophthalmichthys nobilis/molitrix	x	x		x	x	x	x	x	x		x	
Leuciscus aspius	x		x	x	x	x	x	x	x	x	x	
Pseudorasbora parva	x	x	x	x	x	x	x	x	x	x	x	
Rhodeus colchicus	x	x	x	x	x	x	x	x	x	x	x	
Rutilus sp.	x	x	x	x	x	x	x	x	x	x	x	
Squalius orientalis	x	x	x	x	x	x	x	x	x	x	x	
Vimba vimba	x		x	x	x	x	x	x	x	x	x	
Gambusia holbrooki	x		x			x	x					
Esox lucius			x			x						
Pungitius sp.			x			x						
Neogobius fluviatilis	x					x	x				x	
Ponticola constructor	x	x	x	x	x	x	x	x	x	x	x	
Rhinogobius lindbergi				x				x	x	x	x	
Mugil cesphalus	x					x	x					
Trachurus mediterraneus		x										
Perca fluviatilis			x			x		x				
Scomber scombrus				x						x		
Oncorhynchus mykiss	x		x	x	x	x	x	x	x	x	x	
Salmo labrax	x						x		x	x	x	
Silurus glanis	x			x				x	x	x		
Lampetra ninae					x							

In addition, DNA of the ninespined stickleback – *Pungitius* was also detected at sampling locations 3 and 6 (Fig. 1) with over 98% similarity to *P. pungitius*. These sequences belong to a taxon that apparently has never been detected in the Georgian aquatic environment and, thus, is a new species record for the country.

The detected taxonomic diversity showed a positive relationship with the water sample size (Fig. 2). In particular, less than 400 ml water resulted in an

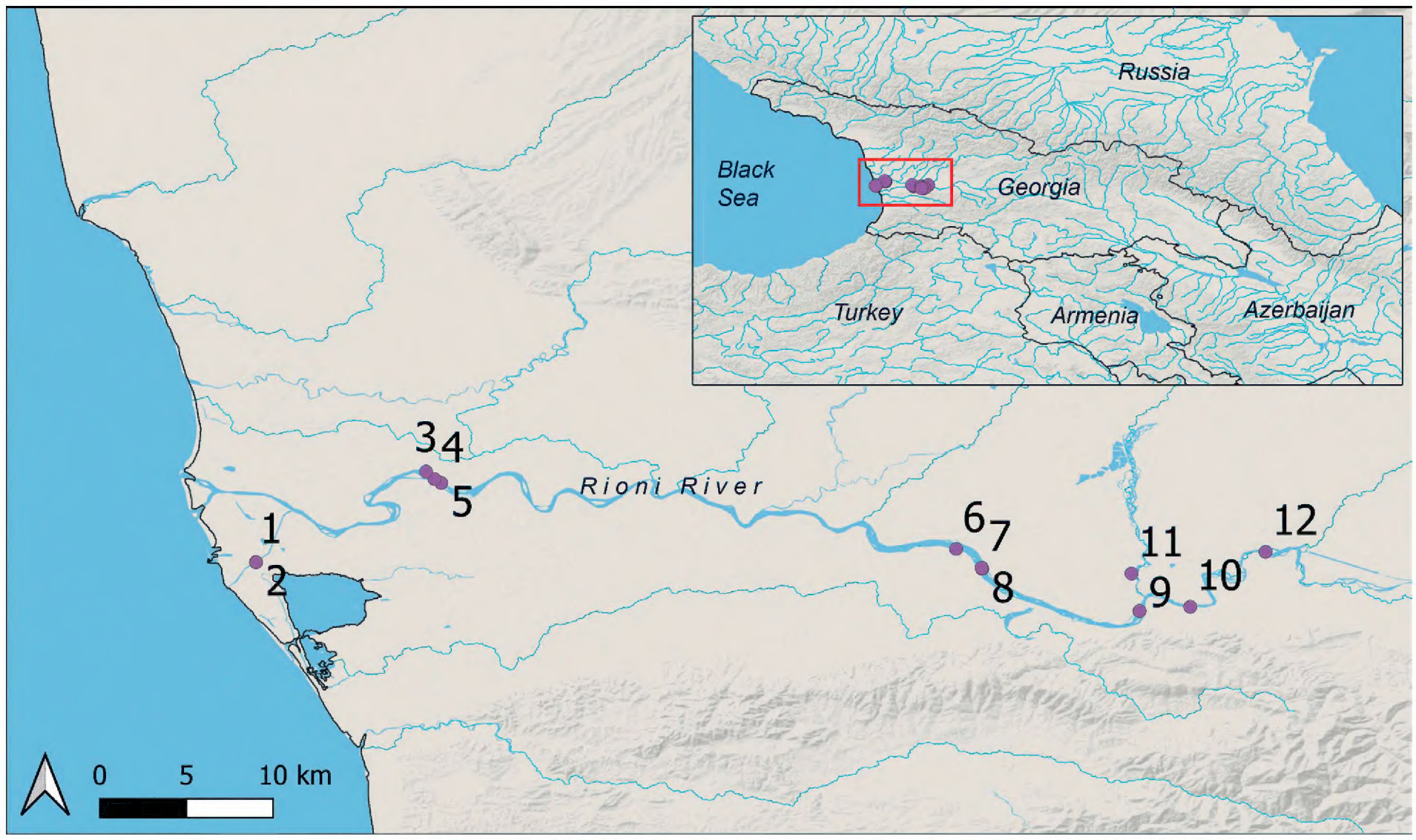

Figure 1. Sampling locations on the Rioni River. Note that samples 1 and 2 are collected from the same site, albeit at different times. Inset map shows the Caucasus region for context.

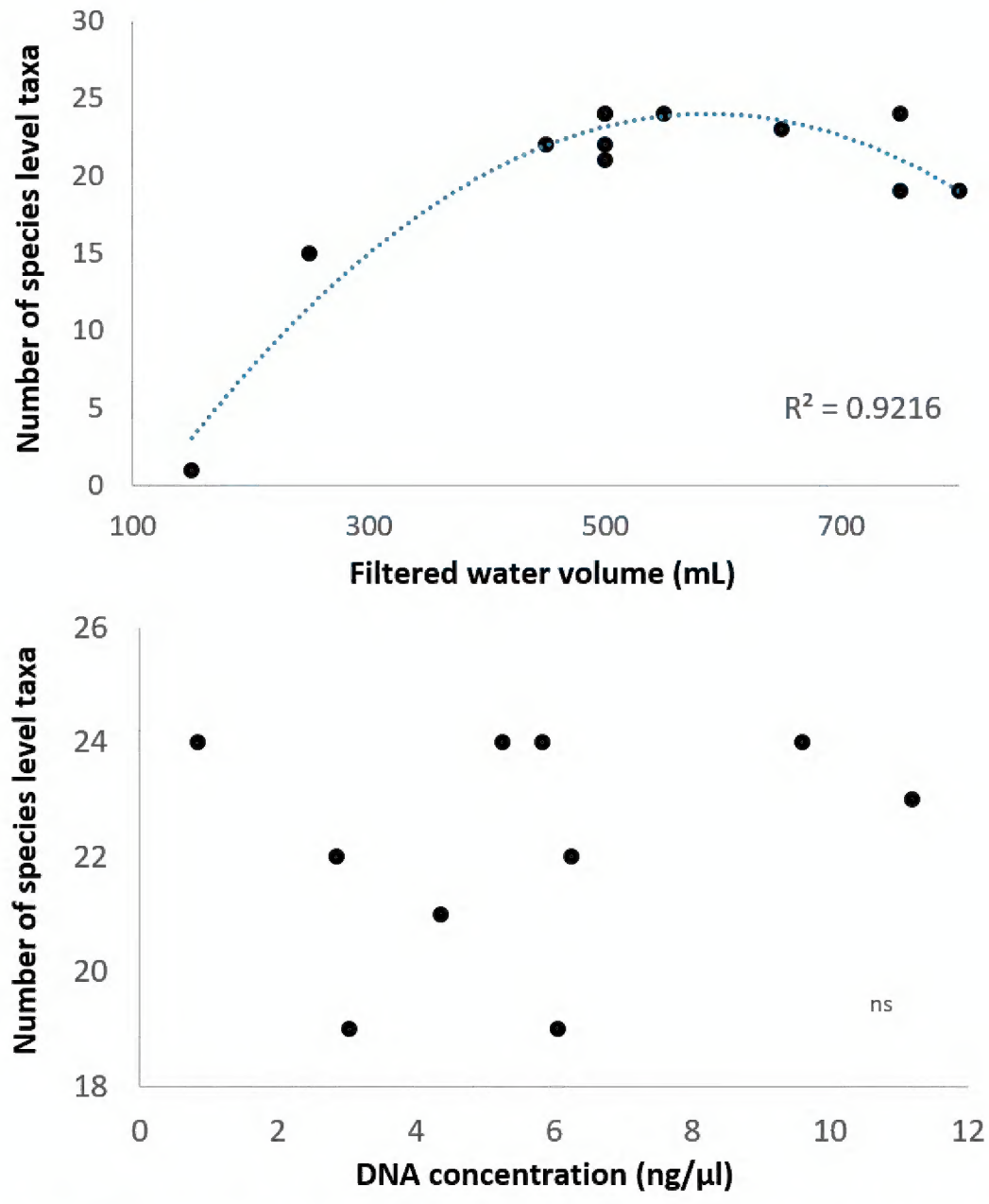

Figure 2. Dependences of detected species number on the water volumes filtered (upper panel) and the DNA concentration in filtrates (lower panel). Note that the concentration of DNA for first and second samples is not included in the graph on the lower panel, because inexact numbers (i.e. > 20) were indicated in the report.

apparent drop in detected taxonomic diversity. On the other hand, no further increase in diversity is evident above the 500 ml volume of filtered water. In contrast, eDNA concentration did not have any visible effect on taxonomic diversity (Fig. 2).

Discussion

Fish fauna of the Rioni River

The first (and only) systematic investigation of the fish fauna of the Rioni River was carried out by (Elanidze 1956), who reported records for 46 species-level taxa. Considering the synonymy due to outdated nomenclature given in that publication, the actual species list given by (Elanidze 1956) is equivalent to 41 currently-recognised species (Table 2). It is noteworthy that a systematic study of the ichthyofauna of the Rioni River has not been conducted since then, with only a few publications reporting new findings, including reports of *Lampetra ninae* and *Anguilla anguilla* from the Rioni River Basin (Elanidze 1983). In addition, very recently, two new species were added to the Rioni River fish list, including the newly-described species – *Oxynoemacheilus phasicus* (Freyhof et al. 2021) mentioned by (Elanidze 1956) as *Nemachilus* sp. and Epitashvili et al. (2020) as *Oxynoemacheilus* sp. – and one alien species – *Gymnocephalus cernua* (Epitashvili et al. 2020). Thus, prior to our current study, 44 species were known for the Rioni River (Table 2).

Careful examination of the eDNA data provides evidence of at least nine additional species in the Rioni River. This includes three invasive alien species: *Carassius gibelio*, *Pseudorasbora parva* and *Rhinogobius lindbergi*, which are widespread and generally abundant in the South Caucasus Region (Shoniya et al. 2011; Japoshvili et al. 2013, 2020; Kuljanishvili et al. 2021). As already reported by Kuljanishvili et al. (2021), *R. lindbergi* is a recent introduction for western Georgia (and for the eastern Black Sea Basin). This small-bodied species is a cryptic invader and its discovery is rather difficult due to morphological similarities with native gobies. This species was also detected with the help of DNA barcoding in eastern Georgia (Epitashvili et al. 2020; Japoshvili et al. 2020). Finding *R. lindbergi* in five sampling locations out of 12, indicates that the species is already widely established in the Rioni River Basin. Most probably, the species is already in other eastern Black Sea rivers, for which additional research is needed.

The other three alien species from the Xenocyprididae family, such as *Ctenopharyngodon idella*, *Hypophthalmichthys molitrix/H. nobilis* and the salmonid *Oncorhynchus mykiss*, seem to be robustly represented in the Rioni River. Kuljanishvili et al. (2021) indicated that these species are subject to regular stocking in the region and not yet established. At least no self-sustaining populations are known yet. Finding DNA evidence in 6, 9 and 10 sampling locations out of 12 for *C. idella*, *H. molitrix/nobilis* and *O. mykiss*, respectively and, in some cases, a dominant proportion of total eDNA, indicates a significant presence of these species within the study area. However, further research is needed to clarify eDNA sources and evaluate how established these populations are in the River. Nevertheless, the invasive status and the high risks of establishment related to all these non-native species as suggested by Kuljanishvili et al.

(2021) and Mumladze et al. (2022) are fulfilled and, thus, care must be taken to prevent or mitigate the potential threats for the native fauna and ecosystems.

Perhaps the most interesting finding in this study is the detection of the DNA sequence of *Pungitius pungitius*. This species is usually known from the northern regions of Eurasia and America (Kottelat and Freyhof 2008). From the northern Black Sea and Azov Sea regions, another species *P. platygaster* is known that was not previously recorded from the south and eastern Black Sea regions. Based on our results, we cannot confidently say if the sequences in our samples belong to this latter species instead. While the detection of *Pungitius* in Georgia is a new country record, further study is needed to resolve the species identity.

Lastly, the DNA detection of three marine species in the Rioni River – Atlantic mackerel (*Scomber scombrus*), anchovy (*Engraulis encrasicolus*) and Mediterranean horse mackerel (*Trachurus mediterraneus*) is not very surprising. On the one hand, these species can be considered contaminants since they are the main market fish widely available all along the Rioni River settlements. Thus, there is a chance that these commercially targeted species DNA in the river arrived via wastewater effluent. Furthermore, they are often sold and consumed in the Rioni River area and nearby communities. On the other hand, all three species are suggested to frequently migrate at the lower reaches of the Rioni River (Elanidze 1956, 1983) and, thus, the occurrence of their DNA at sampling localities close to the river mouth (one and two localities on the map) could be a sign of their actual presence.

Fresh and brackish water fish DNA library and eDNA-based detection efficiency

From the 34 taxa discovered amongst the sampled eDNA reads, 17 (51%) taxa were correctly identified to species level. Identification ambiguity related to the remaining 17 taxa is mainly due to gaps in the barcode reference library, while in a few cases, unresolved taxonomy also played a role. For instance, species complexes of roaches (*Rutilus*) or Caucasian gobies (Gobiidae) are still waiting for comprehensive investigation. The current CO1 (Cytochrome Oxidase 1) barcode library for Georgian fresh and brackish water fishes includes only 52% of species at the time of writing this article (excluding taxa that are usually considered marine species, for example, *T. mediterraneus*, *E. encrasicolus*, *S. scombrus*) (Epitashvili et al. 2020) and the 12S marker library is likely to be much less complete. Thus, the eDNA-based discovery of 32 fresh/brackish water species, of which 51% were correctly identified at species level, is in line with the current development of the regional fish DNA barcode reference library.

Species that were not detected during our eDNA survey, but are historically known for the Rioni River (e.g. Elanidze (1956)) fall into three categories. First are the rare/threatened species, populations that have either declined in recent decades likely due to anthropogenic influence (e.g. *Acipenser* spp.) or naturally occur in very low population densities in the rivers (*Anguilla anguilla*, *Blicca bjoerkna*). The continued existence of some threatened species in the Rioni River is questionable. For instance, *A. nudiventris* was considered locally extinct in the River until recent targeted field-based sampling revealed the presence of at least three species of sturgeons Stellate Sturgeon, Russian Sturgeon and Ship Sturgeon in the Rioni River (Beridze et al. 2022a, b). The second category

includes species that are brackish-water species (*Alosa tanaica*, *Platichthys flesus*, *Syngnathus abaster*, *Chelon* spp., *Atherina caspia*) occurring in rivers (usually near the river mouth) with low densities (Elanidze 1956). The third category includes species that are predicted for the region and which should occur in the Rioni River, for example, *Neogobius melanostomus*, *Petroleuciscus borysthenicus*, *Oxynoemacheilus phasicus* and *Sander lucioperca* (Elanidze 1956, 1983; Ninua and Japoshvili 2008). Small-bodied *O. phasicus* is widespread in the middle part of the Rioni River and its tributaries, but no dense populations have been reported (Freyhof et al. 2021), nor is the species known to range in the lower reaches of the River. Thus, these species might not inhabit the sampling area. Similar arguments are hard to devise for the other three species, the absence of their DNA might be indicative of the insufficiency of sampling, either because the locations were not adequate or the volume of water was insufficient—in other words, improper sampling strategies related to fish life history. Thus, the potential reasons for lack of detection within these three categories could be due to: (1) the species might not inhabit the sampling area or might simply not have been active in that environment during sampling; (2) volume of water was insufficient when sampling; and (3) sample size was small.

Concluding remarks

In spite of some complications, such as a poorly-developed DNA barcode reference library, limited sampling (only 12 samples, all from the lower parts of the river and limited coverage of the depth gradient) and small volumes of water filtered per sample, the eDNA survey recovered more than 70% of the known fish taxa and also detected new invasive and market species. Although the study lacks true field replicates and field controls which limit our ability to interpret the data, we show that eDNA is very effective in assessing fish species assemblages in the Rioni River and the methodology has great potential as a means to assess fish communities either for species inventory or monitoring purposes.

Acknowledgements

We would like to thank Fauna & Flora International for their partnership throughout this process, especially the FFI Caucasus Programme and their Sturgeon Conservation Team: former sub-regional manager Fleur Scheele, Michelle Klailova, Bianca Roberts, Mikheil Potskhishvili and FFI's Conservation Officer Janeli Rogava as well as the local fishers. We also thank to Giorgi Epitashvili (Institute of Zoology of Ilia State University) for his advice regarding the Rioni fish. Lastly, we would like to thank the reviewers and editor (Dr. Katy Klymus) of the journal for their helpful suggestions that significantly improved the manuscript.

Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statement

No ethical statement was reported.

Funding

No funding was reported.

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Data availability

All of the data that support the findings of this study are available in the main text.

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